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Biosynthesis of Zn-doped $CuFe_2O_4$ nanoparticles and their cytotoxic activity

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Zn-doped $CuFe_2O_4$ nanoparticles (NPs) were eco-friendly synthesized using plant extract. These nanoparticles were characterized by X-ray diffraction, Fourier-transform infrared spectroscopy, scanning electron microscope (SEM), energy-dispersive X-ray spectroscopy and thermal gravimetric analysis (TGA). SEM image showed spherical NPs with size range less than 30 nm. In the EDS diagram, the elements of zinc, copper, iron, and oxygen are shown. The cytotoxicity and anticancer properties of Zn-doped $CuFe_2O_4$ NPs were evaluated on macrophage normal cells and A549 lung cancer cells. The cytotoxic effects of Zn-doped $CuFe_2O_4$ and $CuFe_2O_4$ NPs on A549 cancer cell lines were analyzed. The Zn-doped $CuFe_2O_4$ and $CuFe_2O_4$ NPs demonstrated IC_{50} values 95.8 and 278.4 µg/mL on A549 cancer cell, respectively. Additionally, Zn-doped $CuFe_2O_4$ and $CuFe_2O_4$ NPs had IC_{80} values of 8.31 and 16.1 µg/mL on A549 cancer cell, respectively. Notably, doping Zn on $CuFe_2O_4$ NPs displayed better cytotoxic effects on A549 cancer cell compared with the $CuFe_2O_4$ NPs alone. Also spinel nanocrystals of Zn-doped $CuFe_2O_4$ (~13 nm) had a minimum toxicity (CC_{50} = 136.6 µg/mL) on macrophages J774 Cell Line.

Nanotechnology is a field of science and technology in which small dimensions in the range of nanoscale play a crucial role on this science[1–3]. Nanotechnology involves the production and use of particles at the size scale of molecules and intracellular structures[4,5]. Nanoscale is commonly considered to deal with particles in the size range < 100 nm (at least in one dimension), which called nanoparticles[6–8]. Nanostructures have been employed in all different fields of science and technology such as nanomedicine[9], gene/drug delivery[10], energy[11,12], agriculture[13–16], and even space[17]. Thus, the current growing trends show that nanotechnology is playing an important role in the scientific revolutions. Recent developments in science[18–28] and technology[29–39] even in engineering[40–42], epidemiology[43–49], mathematics[50–54] and geometry[55–58] have significant impact on human health[59–61] and life[62–68]. Nanoparticles (NPs) with different shapes[69–73] and sizes have been widely fabricated via a large number of physicochemical and bio-based synthesis techniques[74], including electron irradiation, chemical reduction[75,76], sol gel[77], microwave-assisted synthesis[78], and plant-mediated synthesis techniques[79–82]. However, there are still several challenging issues regarding their stability, aggregation/sedimentation, size distribution, and control of morphology[83–85].

The synthesis of NPs with unique physicochemical properties and multifunctionality are among the topics of interest for researchers[86–88]. Multimetallic NPs have recently received attention in medical and biomedical fields[89]. These NPs have illustrated suitable stability, multifunctionality, and applicability for various clinical and biomedical appliances[90]. Among them, magnetic copper ferrite ($CuFe_2O_4$) NPs as spinel ceramic materials[91] demonstrated suitable antioxidant effects and good biodegradability. Spinel ferrites have the general formula of "MFe_2O_4" where "M" represents divalent cation (Zn, Cu, Mn, Co, Mg, Ni, etc.)[92]. Additionally, these NPs can be utilized for cellular labeling, hyperthermia, and anticancer applications. Copper ferrite NPs caused liver HepG2

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cancer cells necrosis (in vitro) by increasing the oxidative stress and caspase-3 activity[1]. Also, these multimetallic magnetic particles have low production costs, and can be recycled in water treatment[90,93].

Magnetic zinc ferrites ($ZnFe_2O_4$) are recyclable and biocompatible catalysts with high anti-inflammatory activity[94]. Zinc ferrite NPs demonstrated good biocompatibility and hemocompatibility with human dermal fibroblast cells (HDF) and red blood cells (RBC), respectively. On the other hand, they have high toxicity against Gram-positive and Gram-negative bacteria by increasing reactive oxygene stress (ROS)[95]. Ferrite multi-metals such as nickel zinc ferrite and chromium copper ferrite have shown promising clinical and biomedical applicability due to their unique physicochemical features. The antibacterial properties of chromium copper ferrite NPs are greater than those of copper ferrite NPs. With the addition of chromium metal, the surface-to-volume ratio in chromium copper ferrite NPs was increased, and these NPs had more damaging activity against bacterial membranes[96]. In vitro studies demonstrated that nickel zinc ferrite NPs had time-dependent and concentration cytotoxicity against colon HT29, breast MCF7, and liver HepG2 cancer cells. They could increase the apoptosis of cancer cells by mitochondrial and chromosomal damages. Maximum cell death in liver cancer cells was at a concentration of 100 µg/mL, and also it was observed in colon and breast cancer cells at a concentration of 1000 µg/mL[97].

Herein, for the first time, Zn-doped copper ferrite (Zn-doped $CuFe_2O_4$) NPs were eco-friendly synthesized using plant extracts. Nasturtium extract was utilized as the main precursor for the synthesis of nanostructures with low toxicity and high stability. Physicochemical properties of nanostructures synthesized by applying *Nasturtium officinale* extract were evaluated by X-ray powder diffraction (XRD), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), Fourier-transform infrared spectroscopy (FTIR), and thermal gravimetric analysis (TGA). In vitro studies of Zn-doped copper ferrite nanostructures against A549 human lung adenocarcinoma cells were performed based on 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method.

Materials and methods

Materials and cell lines. Tetrazolium dye (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), and 1% penicillin–streptomycin solution were procured from INOCLON (Tehran, Iran). Fetal bovine serum (FBS) was purchased from Biochrome (Berlin, Germany). Ferric nitrate ($Fe(NO_3)_3.9H_2O$, ≥ 98%), zinc nitrate ($Zn(NO_3)_2{\cdot}6H_2O$, 98%), and copper (II) chloride ($CuCl_2{\cdot}2H_2O$, ≥ 99.0%) salts were purchased from Sigma-Aldrich Company. All the steps were performed under sterile conditions. Deionized water was utilized in all stages. A549 human lung adenocarcinoma cancer cells and murine macrophage cell line (J774-A1) were obtained from the Pasteur Institute of Iran's (Iran) cellular bank. Cells were cultivated in DMEM medium supplemented with 10% FBS, 1% antibiotic mixture (penicillin/streptomycin), and maintained at humidified atmosphere under standard conditions (37 °C, 5% CO_2).

Plant-mediated synthesis of Zn-doped $CuFe_2O_4$ NPs. The young leaves of the Nasturtium plant were washed with deionized water. The surface moisture of the leaves was removed at 27 °C and turned into a soft powder. 1 g of plant powder was mixed by 10 mL of deionized water and stirred at room temperature for 24 h. The plant extract was filtered by Whatman filter paper (the size No. 40) and centrifuged. $Fe(NO_3)_3{\cdot}9H_2O$ (1.7 g), $Zn(NO_3)_2{\cdot}6H_2O$ (0.8 g), and $CuCl_2{\cdot}2H_2O$ (0.8 g) salts were added to 21 mL of plant extract and dissolved at room temperature under vigorous stirring, respectively. After complete dissolution of salts, the pH of the mixture was increased from 4 to 7 by adding NaOH 1 M under the same conditions. After that, 15 mL of deionized water was added dropwise to the mixture and sterilized continuously for 2 h at room temperature. The resulting mixture was transferred to an autoclave and placed in an oven at 170 °C for 13 h. The synthesized NPs were washed several times with deionized water. Finally, the obtained powder was dried at 80 °C for 10 h and calcined at 400 °C for 4 h.

Cytotoxic effects of Zn-doped $CuFe_2O_4$ NPs on macrophages J774 cell line. For the cytotoxicity analysis of NPs on macrophages J774 cell line, we determined the CC_{50} (cytotoxicity concentration for 50% of cells) for various concentrations (1, 5, 10, 50, 100, 500, and 1000 µg/mL) of Zn-doped $CuFe_2O_4$, ZnO[98], CuO[99], and $CuFe_2O_4$ NPs on macrophages. Macrophage cells were plated at 10^6 cells/mL in 96-well Lab-Tek (Nunc, USA) and left to adhere for 24 h at 37 °C and 5% CO_2. After removing the non-adherent cells by washing with DMEM medium, the cells were incubated at similar conditions as mentioned before. Thereafter, 190 µL of complete DMEM medium was added in each well, and after that 10 µL of NPs dilution was added (as previously prepared in medium). Macrophages were preserved with the NPs from 1 to 1000 µg/mL for 72 h. The cytotoxicity rate was evaluated using the WST1 colorimetric cell viability assay as previously defined in the promastigote sensitivity assay. All experiments were performed in triplicate similar to the previous stages[100].

Cytotoxicity analysis of Zn-doped $CuFe_2O_4$ NPs against cancer cells. The cytotoxicity of Zn-doped $CuFe_2O_4$, ZnO, CuO, and $CuFe_2O_4$ NPs (various concentrations: 1, 5, 10, 50, 100, 500, and 1000 µg/mL) against A549 lung cancer cells was measured based on MTT assay for 72 h. 10^4 cells/cm^2 were seeded in 96-well plates. After attaching the cells to the plate wall, different concentrations of NPs were added and incubated at 37 °C with 5% CO_2 for 72 h. After this procedure, the cells were washed with phosphate buffer saline (PBS), and the medium was discarded. In the following, 5 mg/mL of MTT dye in PBS was applied to each well, and the plate was incubated for 4 h. 100 µL of DMSO solution was added to each well, and then stored in the dark place at 25 °C for 15 min. Finally, using a microplate reader, the absorbance of dissolved formazan was measured at 570 nm (DYNEX MRX, USA). The proportion of viable cells to untreated cells was deployed to characterize the

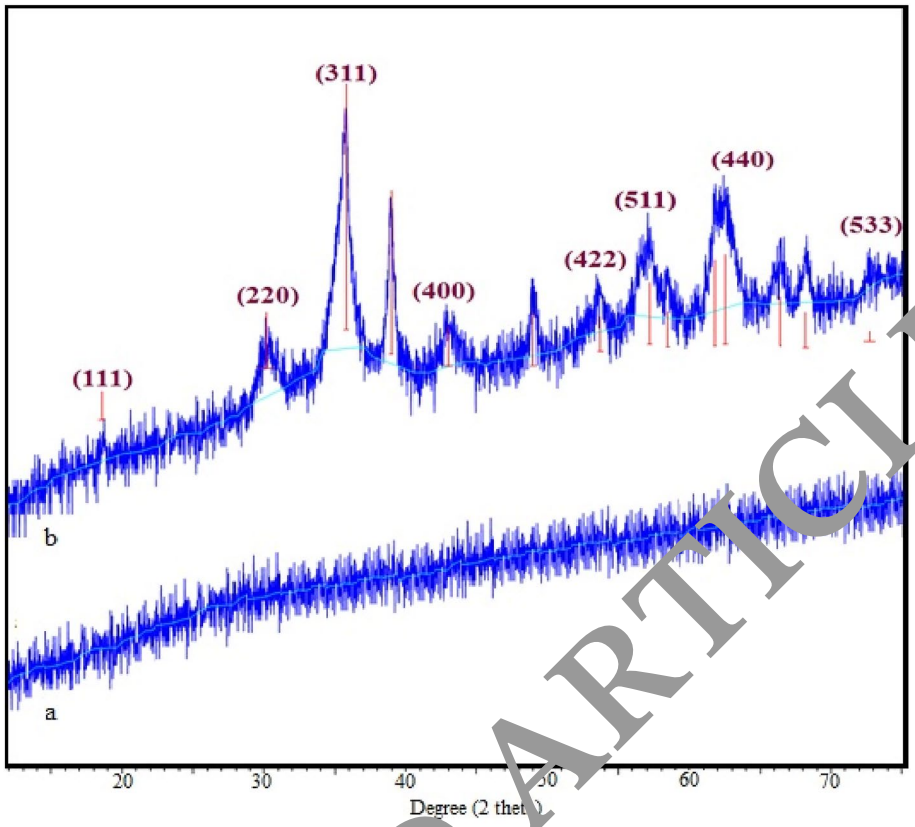

Figure 1. XRD diagram of plant extract (**a**) and Zn-doped $CuFe_2O_4$ NPs (**b**).

relative viability of A375 cells. The inhibitory concentration needed for 50% and 80% cytotoxicity (IC_{50} and IC_{80}) was assessed by applying the Probit test and plotting the level of inhibition vs. the concentration.

Results

The XRD analysis was performed using an X'PertPro (Panalytical Company, Holland) diffractometer with wavelength of X-ray beam 1.5 Å and Cu anode material. XRD measurements were performed to determine the crystalline phase and nature of biogenic nanostructures (2θ range from 10° to 80°). XRD data of plant extract and nanostructures are depicted in Fig. 1a,b. The presence of strong peaks in 2θ range 35.7°, 62.5°, and 39° confirmed the crystalline phases of copper-ferrite ($CuFe_2O_4$)[101] and zinc-doped copper ferrite (Zn doped $CuFe_2O_4$) NPs in the synthesized NPs, respectively. The reflection planes 111 (18.5°), 220 (30°), 311 (35.7°), 400 (43°), 422 (53.5°), 511 (57°), 440 (62.5°), and 533 (72.5°) verified the spinel crystallites phase[102] of Zn-doped $CuFe_2O_4$ as described previously[103,104].

In the XRD pattern, the reflection (311) is the most intense peak. The lattice constant was calculated using the interplanar spacing distance and the respective (hkl) parameters using the following relation[105]:

$$a - \frac{\lambda\left[h^2 + k^2 + l^2\right]^{1/2}}{2\sin\theta} \cdot \mathring{\mathrm{A}}$$

The crystallite size was estimated from the most intense peak of XRD data (311). The crystallite size was calculated as a function of Zn content x using Debye–Scherrer's formula ($D = 0.9\lambda/\beta \cos\theta$). In this formula "λ" is the wavelength of the X-ray radiation, "β" is the full-width half maximum and "2θ" is the diffraction angle. As a result, the crystallite size of NPs was found to be ~ 20 nm.

FTIR analysis of Zn-doped $CuFe_2O_4$ NPs in the range of 300 to 4000 cm^{-1} with KBr pellet was performed by tensor II (Bruker Company, Germany) device. FTIR analysis identified the functional groups and chemical bonds present in the synthesized NPs (Fig. 2). Peaks 476, 551, and 1049 cm^{-1} established the stretching bond of O atom in the $CuFe_2O_4$ structure[106,107]. The 551 and 1049 cm^{-1} broad peaks were attributed to the octahedral spinel structure of $CuFe_2O_4$ NPs. The weak peak transfer of 476 cm^{-1} to the two regions 551 and 1049 cm^{-1} confirmed the transfer of the O stretching bond from the tetrahedral location to the octahedral location[108,109]. The peaks of 3449 and 3346 cm^{-1} can be attributed to the stretching vibration of O–H group of nasturtium (plant) phenolic compounds. It was revealed that phenolic compounds of plants played a reducing role for the synthesis of metal NPs[110].

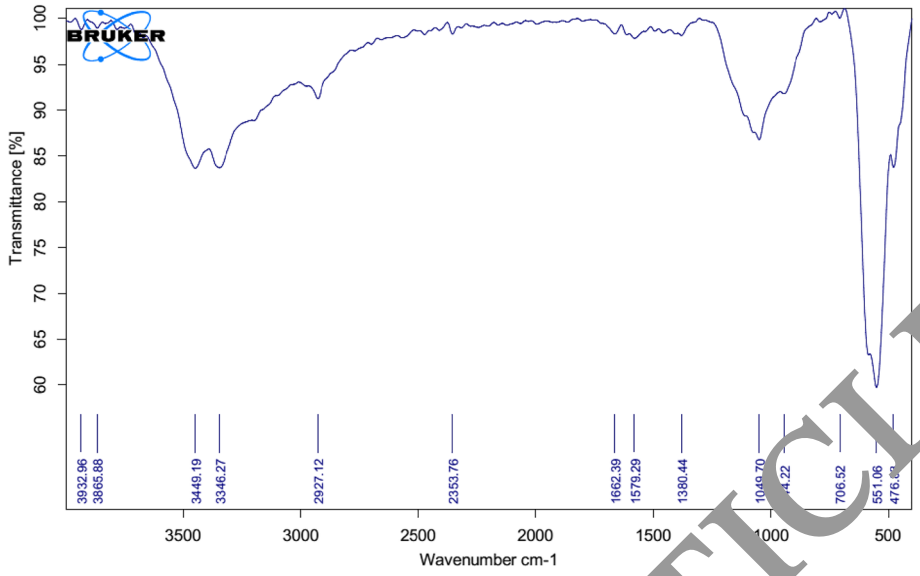

Figure 2. FT-IR spectra of Zn-doped $CuFe_2O_4$ NPs.

Elemental composition and morphology evaluations of Zn-doped $CuFe_2O_4$ NPs were performed using FESEM-EDS. Surface images with a magnification of 100 Kx (Fig. 3a) and components (Fig. 3b) of the Zn-doped $CuFe_2O_4$ were obtained using Sigma VP, ZEISS Company equipped with EDS detector of Oxford Instruments Company. SEM image with bright-field background demonstrated spherical NPs with size range less than 30 nm. In the EDS diagram, the elements of zinc, copper, iron, and oxygen are shown. The presence of Cu, Zn, Fe and O elements in EDS spectra confirmed the formation of deposited Zn-doped $CuFe_2O_4$ spinel ferrite. The elemental composition of all samples was correlated to the stoichiometric theoretical composition of Zn-doped $CuFe_2O_4$.

Thermal analysis of not calcinated Zn-doped $CuFe_2O_4$ NPs was performed to investigate the formation of the spinel ferrite phase of the prepared spinel ferrite, as previously described[111]. Changes in the physical behavior of Zn-doped $CuFe_2O_4$ NPs were evaluated using TGA based on temperature and time using TG 209 F3Tarsus®, NETZSCH Germany Company device (Fig. 4). TGA and DTA evaluations of the NPs were performed under N_2 atmosphere at the heating rate of 10 °C/min within the temperature range 25–800 °C. Weight loss at about 200 °C was attributed to the decomposition of metal hydroxide and the crystallization of Zn-doped $CuFe_2O_4$ NPs[112].

Anticancer properties of Zn-doped $CuFe_2O_4$ NPs. The cytotoxicity properties of Zn-doped $CuFe_2O_4$ NPs were evaluated on macrophage normal cells and A549 lung cancer cells for 72 h, respectively. On the other hand, for better evaluation of anticancer effects of the components in Zn-doped $CuFe_2O_4$ NPs, the aforementioned tests were performed on ZnO, CuO, and $CuFe_2O_4$ NPs. Results obtained from cytotoxicity analysis of Zn-doped $CuFe_2O_4$, ZnO, CuO, and $CuFe_2O_4$ NPs on murine macrophages, with CC_{50} values of 136.6, 762.36, 98.5, and 309.3 µg/mL, are shown in Fig. 5a, respectively. According to CC_{50} values, Zn-doped $CuFe_2O_4$, ZnO, and $CuFe_2O_4$ NPs displayed no significant cytotoxic effects against macrophage cells, but CuO NPs illustrated significant cytotoxic effects against normal macrophage cells. Based on our results, Zn-doped $CuFe_2O_4$, ZnO, and $CuFe_2O_4$ NPs were safer for mammalian cells. According to the results, CuO NPs caused oxidative stress and genetic toxicity in mammalian normal cells[113,114]. The cytotoxic effects of Zn- doped $CuFe_2O_4$, ZnO, CuO, and $CuFe_2O_4$ NPs exposed to 1–1000 µg/mL on A549 cancer cell lines are shown in Fig. 5b. The Zn-doped $CuFe_2O_4$, ZnO, CuO, and $CuFe_2O_4$ NPs demonstrated IC_{50} values 95.8, 113.1, 120.2, and 278.4 µg/mL on A549 cancer cell, respectively. Additionally, Zn- doped $CuFe_2O_4$, ZnO, CuO, and $CuFe_2O_4$ NPs had IC_{80} values of 8.31, 12.81, 8.7, and 16.1 µg/mL on A549 cancer cell, respectively. According to the results, these NPs had anticancer properties against lung cancer cells. Due to the high toxicity of CuO NPs against normal macrophage cells, these NPs are not suitable therapeutic agents. On the other hand, further evaluations demonstrated that ZnO NPs had significant toxicity against A549 cancer cells at 31.2 µg/mL. Consequently, the toxicity of ZnO NPs depends on the concentration, time, and size of the NPs[115]. ZnO NPs were synthesized using *Mangifera indica* and illustrated good anticancer properties against A549 cancer cells[116]. Additionally, CuO NPs were eco-friendly fabricated using *Ficus religiosa*, showing desirable anticancer properties against A549 cancer cells with increased apoptosis[117].

Discussion

In this study, Zn-doped $CuFe_2O_4$ NPs were synthesized using *N. officinale* medicinal plant extract. The physicochemical properties of the NPs were determined by XRD, ETIR, SEM, EDX and TGA analysis. The biocompatibility and anticancer properties of the NPs and their components (ZnO, CuO, and $CuFe_2O_4$ NPs) were evaluated against macrophages J774 Cell Line and A549 lung cancer cells, respectively, for 72 h. XRD and FTIR evaluation of Zn-doped $CuFe_2O_4$ NPs confirmed two crystalline phases of $CuFe_2O_4$ and Zn-doped $CuFe_2O_4$. The elements

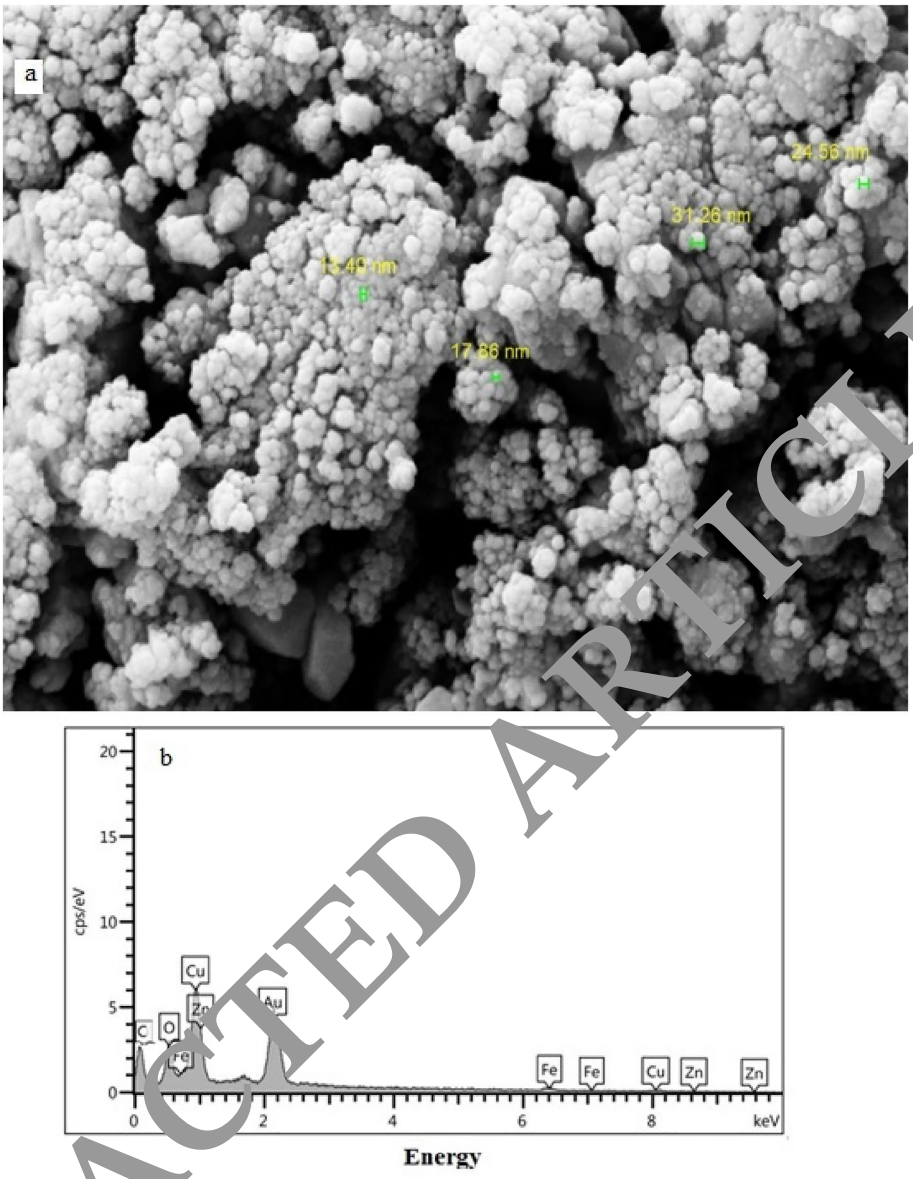

Figure 3. FESEM-EDS analysis: (**a**) SEM image (**b**) EDS diagram of Zn-doped $CuFe_2O_4$ NPs.

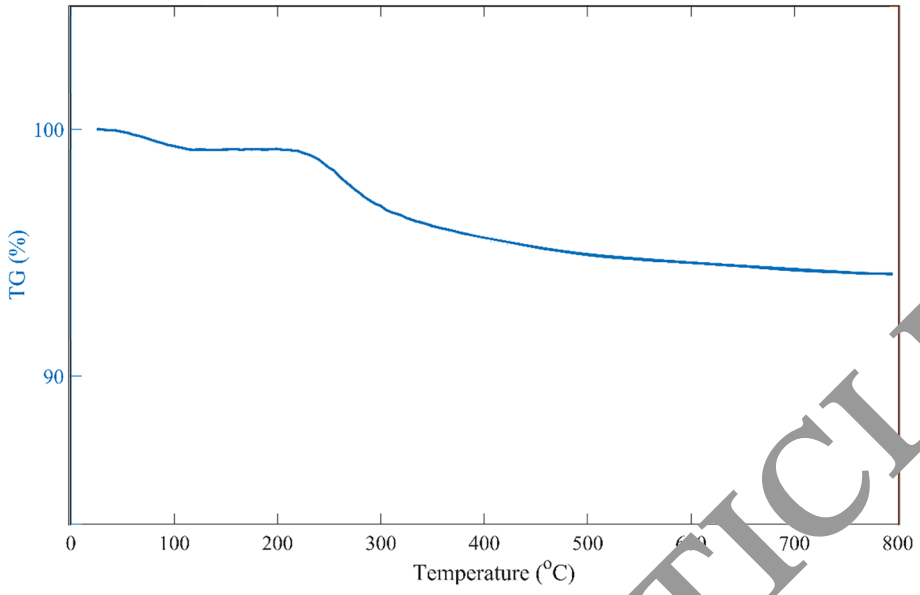

Figure 4. TGA curves of Zn-doped $CuFe_2O_4$ NPs.

(carbon, zinc, copper, iron, and oxygen) of the synthesized spherical NPs were approved by EDS analyses. According to IC_{50} data, Zn-doped $CuFe_2O_4$ NPs had the highest anticancer properties. According to the results obtained from anticancer tests, ZnO and CuO NPs exhibited an increased A549 cell mortality. However, CuO NPs had high toxicity on macrophages normal cells. In recent decades, the application of biogenic NPs together with the phenolic compounds of medicinal plants can be considered as an attractive alternative for the treatment of cancers. *N. officinale* (family: brassicaceae) is an aquatic plant that has significant amounts of iron, calcium, folic acid, glucosinolates, and vitamins C and A. This medicinal plant has significant anticancer and antioxidant properties due to its phenolic compounds[118]. Methanolic extract of this plant has been shown to increase A549 cancer cell mortality by activating apoptotic agents[118]. On the other hand, multimetallic NPs have been focused by researchers due to the synergy of metal elements and multifunctionality[119,120]. Additionally, by increasing the phenolic compounds of Nasturtium extract, the antioxidant activity was enhanced with the lowest IC_{50}[121].

Conclusion

Zn-doped $CuFe_2O_4$ nanopowders were successfully synthesized in one step using Nasturtium plant extract. The NPs were characterized by XRD, FTIR, EDS, TGA, and SEM. The biocompatibility and cytotoxicity of Zn-doped $CuFe_2O_4$ NPs were evaluated on macrophages cell Line. Additionally, the anticancer properties of Zn-doped $CuFe_2O_4$ NPs against A549 lung cancer cells were evaluated. As a result, doping Zn on $CuFe_2O_4$ NPs displayed better cytotoxic effects on A549 cancer cells compared with the $CuFe_2O_4$ NPs alone. Also spinel crystallites of Zn-doped $CuFe_2O_4$ (~ 13 nm) had a minimum toxicity (CC_{50} = 136.6 µg/mL) on macrophages J774 Cell Line. The Zn doped $CuFe_2O_4$ are multi-metallic with suitable applicability and biocompatibility, which should be further studied particularly for the treatment and diagnosis of cancers and infectious diseases. Additionally, these nanomaterials with unique optical and magnetic properties can be considered as attractive candidates for catalytic applications.

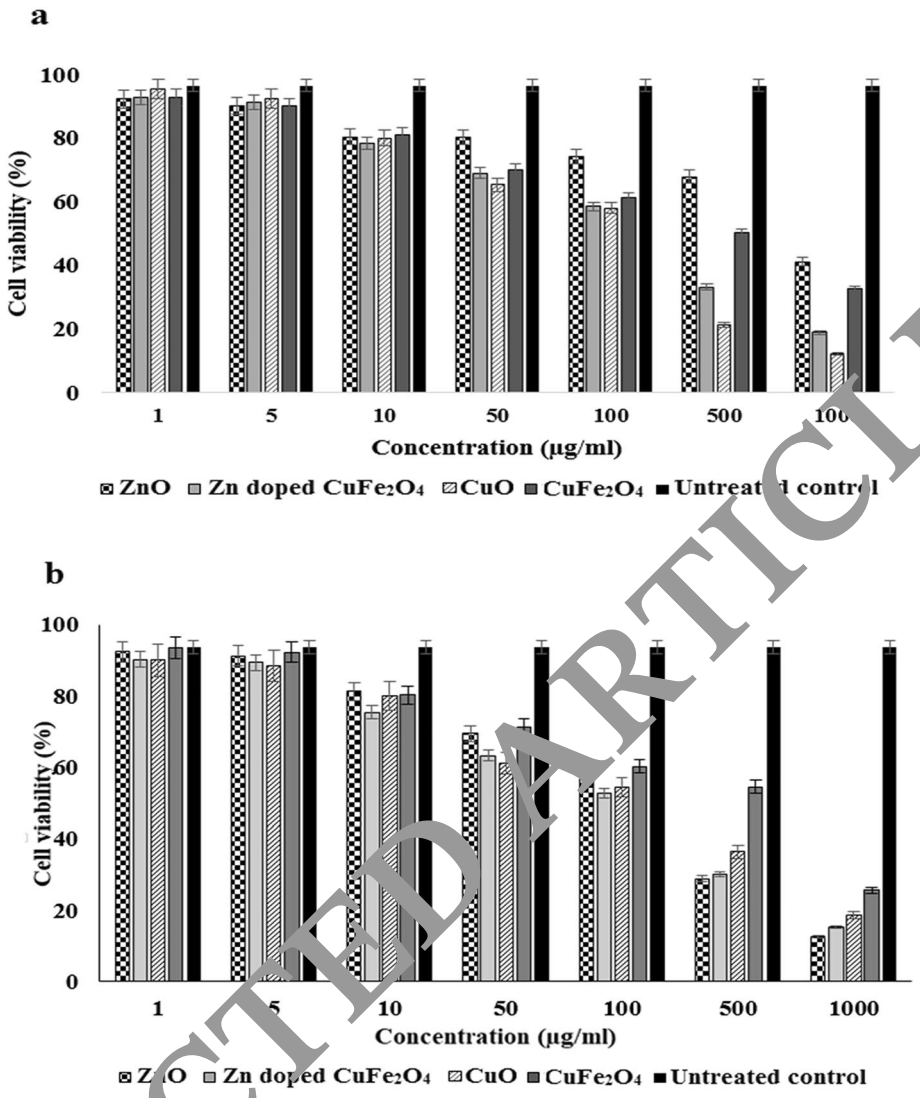

Figure 5. Cytotoxicity analysis: (**a**) the cytotoxicity of NPs against murine macrophages (J774 cells), and (**b**) the cytotoxicity of NPs on A549 lung cancer cells.

Data availability

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

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